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Challenges in electrochemical pre-purification of recombinant proteins from green plant tissues

sgfp produced in tobacco leaves

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The use of recombinant proteins has increased greatly in recent years, as have the number of techniques and materials used for their production and purification. The principal advantage of using plants as bioreactors is the cost of the recombinant protein production, which is about 1000-fold lower as in the case of using CHO cells commonly applied in industry today. Among the different types of "green" bioreactors being studied today, there is a general consensus among scientists that production in green plant tissues such as leaves is more feasible. However, the presence of chlorophyll and phenolic compounds in plant extracts, which can precipitate and denature the proteins besides damaging separation membranes and gels, makes this technology impracticable on a commercial scale. Electrochemically produced aluminium hydroxide gel can be used to adsorb these compounds, and pre-purify recombinant synthetic green fluorescent protein (sGFP) produced in *Nicotiana benthamiana* leaves. Removal efficiencies of 99.7% of chlorophyll, 88.5% of phenolic compounds, and 38.5% of native proteins from the *N. benthamiana* extracts were achieved without removing sGFP from the extracts. Since electrochemical preparation of aluminum hydroxide gel is a cost-effective technique, its use can substantially contribute to the development of future production platforms for recombinant proteins produced in green plant tissues of pharmaceutical and industrial interest.

Introduction

Today the production of biopharmaceuticals is mainly based on two bioreactors: Chinese hamster ovary cell (CHO) and *Escherichia coli*. When roughly comparing the prices of production of these two systems with the production of plant made biopharmaceuticals (PMBs) the one can see, that, the price of production in crops (**Table 1**) is about 10-fold lower than in microbial ant thousand fold lower than in CHO cells.[1] The question arises, why the commercial production today is not based on genetically modified plants.

The reasons for this lie in three different areas: technological, regulatory and business.

Technological. In comparison to the microbial and especially CHO cells the "extracts" obtained in the case of using crops as bioreactors are far more complex, consisting not only of hosts native protein contaminants, but also with secondary metabolites, among which the most deleterious are the phenolics and chlorophyll.[2] Therefore, the most challenging step is to provide the relatively stable extract, which, in addition has to be compatible with chromatographic resins used today. The compatibility of extracts with chromatographic resins has to be assured, since these resins represent the highest cost among the materials used in the DSP of biopharmaceuticals.

Regulatory. PMBs present two major challenges for the regulatory bodies.[3] Regulators of agricultural biotechnology are confronted with novel type of crop use, and drug regulators must deal with a novel drug-production concept. FDAs draft guidance on PMBs in general differs in relatively small amount to the ones regulating the biopharmaceuticals produced in cell lines. Among the others, it requires that the acceptance criteria on product concentration and total protein concentration are established, and that the initial processing of plant material needs to be validated.

Business. The risk of commercial production of PMBs is relatively high, based on two areas. The first is the variability of the crop material produced in comparison to the one produced by CHO and microbial cells. Highly reproducible systems are required in order to produce the pharmaceuticals especially in the case of biopharmaceuticals, where the cost of production are among the highest. The rejection of the batch because of its non-conformity with the process performance characteristics and specifications of the product established during the process

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Table 1. Cost of production of recombinant proteins[1]

Expression system	Cost (USD/g)
Chinese hamster ovary cells (CHO)	300
Transgenic hicken eggs	1–2
Transgenic goat milk	1–2
Microbial fermentation	1,00
Transgenic plants	0,10

Cost of production using corn seeds at expression of 1% of recombinant protein.

Table 2. Zeta potential and diameter of aluminum gel particles formed by electrocoagulation in solutions of 200 mmol/L NaCl at different pH values

	Electrocoagulation pH				
	7.5	**8.0**	**8.5**	**9.0**	**9.5**
Particle diameter (μm)	597	632	659	646	636
Zeta potential (mV)	21.3	13.8	7.1	5.1	3.6

validation could lead to high financial losses to the company performing such tasks. Here again, the reproducible and robust extraction and clarification step, besides being cost effective, will be required.

When summarizing all three areas of challenge one could conclude, that the main challenge lies in the first processing steps which has to be well characterized, predictable and robust, besides being of low cost. However, no process encountered in the literature concerning the clarification (pre-purification) step did take in account the predictability of the process in consideration.

Electrocoagulation in Bioprocesses

The main advantage of the process described in the original article[4] lies in its highly predictable and reproducible—the characteristic that will benefit in process validation. The reason for this high reproducibility lies within the process of production of the aluminum hydroxide gel. More specifically its constant diameter and its superficial charge which can be easily controlled by pH of the solution during aluminum hydroxide gel production (**Table 2**; for more information, see ref. 4). In addition, extraction buffer contained sodium metabisulfite preventing quninine formation and therefore preventing covalent bonding between protein and phenolic compounds, what results in higher phenolics removal and lower protein removal. Higher protein removal in the case when no sodium metabisulfite is present in extraction buffer (data not published), is probable the consequence of phanolics removal, which is bound to protein. Therefore, the addition of the reducing agent, that prevents the quinine formation is essential in the leaf derived biopharmaceuticals is crucial since 10 to 20% higher efficiencies of phenolics removal and at the same time 10 to 20% lower protein removals were observed. From this data we can speculate, that about 10 to 20% of the protein is covalently bound to phenolics if no reducing agent is used during the extractions step. No effect on the chlorophyll removal with the addition of the reducing agent was observed.

Additionally, the removal of phenolics, chlorophyll, and proteins diminished with the lower superficial charge of the aluminum hydroxide gel. This proves, that the aluminum hydroxide acts as an ionic exchanger, which superficial charge can be easily manipulated by the pH of electrocoagulation (**Table 2**).

The electrocoagulation therefore has its potential not only as clarification step but also in substituting the ionic exchange resins in chromatographic columns. Not only in plant based bioprocesses but generally speaking in all bioprocesses. The companies producing the biopharmaceuticals or industrial proteins could therefore produce its own "resin" without being dependent on the continuously more expensive chromatographic resins, which, in many cases, show lot to lot variabilites which can impact the specific process. This is due to the fact that each protein interacts "individually" with the resins in terms of specific and not specific interactions.

Conclusions

In the future the development of predictable first process steps in producing the PMBs will be necessary. This "predictable processes" cannot be achieved by experimental design and process parameter optimization, as is the case of the current researches, but by developing and formulating predictable mathematical models, that take into account the actual phenomenon that is the driving force of the separation itself. This can be achieved by in-depth characterization of the processes employed and in parallel characterization of plant extracts and interaction of its components with the "process" material as well as the interaction of the plant components among them self. Additionally the electrocoagulation can also be applied in place of ionic exchange chromatography, however additional research and development is needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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